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Due to the support of the AFOSR Chronobiology Program, we have been very productive in the past year (1994/1994) with about 25 papers completed. Many of these focus on amino acid transmitters in the SCN and surrounding area. We are pursuing the hypothesis that glutamate and GABA constitute two primary transmitters in the SCN, with glutamate exerting excitatory actions, and GABA exerting depressing actions in the adult SCN. We are using combinations of immunocytochemistry, electron microscopy, molecular biology, and patch clamp recording to study the SCN. Many of our studies are described in detail in the attached detailed description of accomplishments and new findings (Section 4). One exciting new area of our research is the finding that GABA, the most prevalent inhibitiory transmitter in the SCN, actually can exert excitatory actions in developing SCN neurons. As this may occur under certain circumstances in adult neurons, this might be one reason why SCN activity is low during one part of the cycle and high during another. Another very interesting set of studies that is being pursued is the modulation of glutamate and GABA in the SCN by neuropeptide Y, NPY. We find long term changes, suggestive of cellular memory, in the activity of GABAergic and glutamatergic neurons after brief exposure to NPY. This may represent a novel mechanism for phase shifting, an important aspect of the circadian clock which has long term ramifications for work shift performance, jet lag, etc.

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Final

PROGRESS REPORT.

1. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH Sept.1995

From Anthony N. van den Pol,PI

Stanford University

Cronobiology Program, AFOSR

2. Objectives. Objectives have not changed from those described in the inital grant application.

A primary focus has been to study the contribution of amino acid transmitters, GABA and glutamate, to the SCN, using combinations of immunocytochemistry, electron microscopy, whole cell patch clamp recording, calcium digital imaging, and molecular biology. Studies include examination both of the SCN and some of the nearby hypothalamic regions to which projects. We have also begun to study the modulation of amino acid transmitters by other neuroactive agents found in the SCN.

3. Status of effort.

Due to the support of the AFOSR Chronobiology Program, we have been very productive in the past year (1994/1994) with about 25 papers completed. Many of these focus on amino acid transmitters in the SCN and surrounding area. We are pursuing the hypothesis that glutamate and GABA constitute the two primary transmitters in the SCN, with glutamate exerting excitatory actions, and GABA exerting depressing actions in the adult SCN. We are using combinations of immunocytochemistry, electron microscopy, molecular biology, and patch clamp recording to study the SCN. Many of our studies are described in detail in the attached detailed description of accomplishments and new findings (Section 4). One exciting new area of our research is the finding that GABA, the most prevalent inhibitory transmitter in the SCN, actually can exert excitatory actions in developing SCN neurons. As this may occur under certain circumstances in adult neurons, this might be one reason why SCN activity is low during one part of the cycle and high during another. Another very interesting set of studies that is being pursued is the modulation of glutamate and GABA in the SCN by neuropeptide Y, NPY. We find long term changes, suggestive of cellular memory, in the activity of GABAergic and glutamatergic neurons after brief exposure to NPY. This may represent a novel mechanism for phase shifting, an important aspect of the circadian clock which has long term ramifications for work shift performance, jet lag, etc.

4. Accomplishments and new findings (Divided into 13 subsections).

1. Which glutamate receptor genes are expressed in the suprachiasmatic nucleus?

The primary input from the retina to the SCN, and from other brain regions also (Kim and Dudek,1992) appear to use glutamate as a transmitter. This would give glutamate a powerful role in regulating light induced phase shifts of circadian rhythms. Interestingly, a diurnal variation in expression of some glutamate receptors in the SCN has been reported (Gannon and Rea,1994). We have concentrated a significant effort in examining glutamate receptors in the SCN and surrounding hypothalamus.

Ionotropic glutamate receptors. Despite the widespread response to glutamate, autoradiographic receptor binding studies using radiolabeled glutamate and a variety of glutamate agonists had not demonstrated strong receptor binding in hypothalamic regions compared to higher regions of the brain such as cortical structures (Halpain et al,1984). In an analysis of many different brain regions, some of the lowest levels of radiolabeled glutamate binding were found in the hypothalamus, including the SCN (Greenamyre et al,1984). To further determine the identity and cellular location of glutamate receptors that were expressed in the SCN and surrounding hypothalamus, in situ hybridization and Northern blots were used to study the ionotropic subtypes of the glutamate receptor. Widespread expression of AMPA, kainate, and NMDA receptor RNA was found in the hypothalamus with the transcripts the same size and number as found in other regions of the brain. Most of the glutamate receptors subunits studied were expressed in greater amounts in hippocampus than in hypothalamus; GluR5 on the other hand showed a greater expression in hypothalamus, particularly in the SCN, than in hippocampus. In the hypothalamus, GluR1 and GluR2 were among the most widely expressed of the non-NMDA ionotropic receptors. Strong expression of these two was found in the SCN. Other AMPA-preferring receptors such as GluR3 was found in the hypothalamus, but was not found in the SCN. GluR4 was found in the SCN, with stronger expression in the ventrolateral division, the region receiving the highest density of retinal inputs. Kainate preferring receptors were also found in the SCN. One of the highest concentrations of GluR5 was in the SCN, particularly in the ventrolateral region. Weak hybridization of the kainate receptor, GluR6 was found in the SCN. GluR7 was not found in the SCN, but was found in the dorsomedial hypothalamus. The N-methyl d-aspartate receptor, NMDAR1, was detected throughout the hypothalamus, and was strongly expressed in the SCN. With many of the glutamate receptor probes, only scattered cells showed detectable expression of the glutamate receptor mRNA as detected by autoradiographic silver grains over neurons; unlabeled cells were mixed among labeled cells. The expression of many different types of ionotropic glutamate receptors within the SCN suggests that multiple modes of ion channel regulation by glutamate probably operate here, and provides further support for the importance of the excitatory transmitter glutamate in regulation of SCN function (van den Pol et al, 1994A).

In the last few months we have been working with a new antibody against the kainate receptor GluR6 in collaboration with Dr.Craig Blackstone (Harvard Univ.). We find strong GluR6 expression in the SCN. This is true both in the developing brain where GluR6 is found at birth, and in the adult rat. Immunoreactivity is found in the perikarya and some long dendrites of a large number of SCN neurons. We see little immunoreactivity on astrocytes. With electron microscopy, we find peroxidase immunoreactivity on the plasma membrane, often in the area of asymmetrical synaptic specializations.

Metabotropic glutamate receptor mGluR1a. The metabotropic glutamate receptors activate second messenger systems via a G-protein. Activation of the metabotropic receptor leads to increases in phosphotidylinositol (PI) hydrolysis, resulting in formation of diacylglycerol and IP_3 and mobilization of

Ca2+ from intracellular stores (Furuya et al,1989); these second messengers modulate a wide variety of enzymatic pathways, gene expression, and cellular growth and differentiation (Mayer and Miller, 1990; Schoepp et al, 1990; Yuzaki and Mikoshiba, 1992; Shigemoto et al,1992). Stimulation of mGluR1 has also been reported to induce the formation of cAMP and the release of arachidonic acid (Aramori and Nakanishi,1992; Fotuhi et al,1993). A number of genes coding for metabotropic (Masu et al, 1991; Houamed et al,1991; Abe et al, 1992; Tanabe et al, 1992) glutamate receptors have been cloned.

In the developing and adult rat hypothalamus, the expression of the metabotropic glutamate receptor mGluR1 was studied with Northern and Western blot analysis, with immunocytochemistry, and with Ca2+ digital imaging. mGluR1 is coupled to a G protein and activation by glutamate and related agonists leads to intracellular phosphotidylinositol hydrolysis and Ca2+ mobilization. mGluR1 RNA could be detected in embryonic hypothalamus, and by the day of birth and prior to the primary period of synaptogenesis, both mGluR1 RNA and protein were strongly expressed. One of the regions showing the strongest early expression of mGluR1 was the SCN. In parallel experiments with digital imaging of cultured hypothalamic cells, some E18 hypothalamic neurons and many astrocytes after 3 days in vitro showed Ca2+ responses to quisqualate and t-ACPD, and to glutamate in the absence of extracellular Ca2+. A greater number of embryonic neurons responded to NMDA than to agonists of the metabotropic receptor. With increased development time in culture, the number of neurons that responded to metabotropic glutamate receptor agonists increased.

The adult hypothalamus contained widespread mGluR1 immunoreactive neurons, particularly dense in the SCN, and some other regions of the periventricular and anterior hypothalamus. Strongly immunoreactive cells were interspersed among neurons with no immunoreactivity. In developing neurons a diffuse immunostaining appeared along dendrites and somata. With time, beginning in the first week after birth, strongly stained puncta appeared, probably associated with synaptic specializations. These puncta were numerous on dendrites of some adult neurons, and were the most strongly stained regions of neurons. Neurons developing in vitro at low neuron densities showed a development of mGluR1 immunoreactivity similar to that of neurons in vivo, but with a delayed progression of immunostaining. We found no obvious staining of axons or of astrocytes. A strong expression of mGluR1 protein was found in the hypothalamus during the first two weeks of life; this expression was substantially reduced in adults. In contrast, cerebellum showed no reduction in mGluR1 protein in adults. Together these data suggest a complex regulation of mGluR1 during development, with sufficient expression of functional receptors in the developing hypothalamus to modulate morphogenesis and synaptogenesis, and later to play a role in transduction of glutamate signals in the adult. Different regions of the brain showed dramatic differences in the expression of mGluR1 during development (van den Pol et al,1994B).

Metabotropic mGluR5. mGluR5 is the second member of the mGluR1/mGluR5 family of metabotropic glutamate receptors. We have examined the distribution of mGluR5 in the SCN. It is strongly expressed in the SCN and some nearby regions of the hypothalamus, but is only weakly expressed in the neurosecretory neurons of the arcuate, paraventricular, or supraoptic nuclei. The antibody we used does not cross react with the mGluR1, a related metabotropic receptor, as tested with immunostaining and immunoblots of cells transfected with the genes coding for mGluR1 and mGluR5. Outside the hypothalamus, we find immunoreactivity in the striatum, olfactory bulb, hippocampus, and cerebellum (Romano et al, 1995).

Expression in the SCN is found early in development, with strongly immunoreactive cells found as early as the day of birth, suggesting expression at embryonic periods also. With mGluR1, we found no glial expression. In striking contrast, with mGluR5, we found some astrocytes with strong labeling of the their plasma membrane. Staining of neurons and astrocytes was blocked in control experiments where the peptide antigen was pre-adsorbed with the antibody (van den Pol, Romano, Ghosh, In press).

2. Subcellular localization of glutamate receptors.

We have examined the subcellular localization of several glutamate receptors, including AMPA, kainate, metabotropic, and NMDAR1. These studies were aided by the generous donation of highly specific glutamate receptor antibodies from colleagues and collaborators including Drs. R.Wenthold, C.Blackstone, R.Huganir, N.Brose, S.Rogers, S.Heinemann, and C.Romano.

A number of studies were undertaken to examine the location of the metabotropic receptor mGluR1 in hypothalamic cells. In the adult hypothalamus, the staining is most intense at dark puncta on the dendritic

and somatic membranes. Similarly, when we studied hypothalamic neurons in vitro, the strongest staining was at dark puncta suggestive of a synaptic localization (van den Pol et al,1994). To verify if the dark puncta were in fact synaptic in nature, we used electron microscopy and immunoperoxidase to study neurons from the SCN and surrounding anterior hypothalamus. If cells were lightly stained the strongest staining was on the dendritic membrane. Surprisingly, although some of the dark staining regions were associated with synaptic specializations, others were not. Many asymmetrical synapses, thought to be the site of excitatory neurotransmission, were devoid of reaction product, despite the positive labeling of membrane on both sides of the unlabeled synapse (van den Pol,1994).

We have also examined the ultrastructural localization of another Ca2+ mobilizing metabotropic glutamate receptor mGluR5 in the SCN and control areas. In the hippocampus, we find immunoreactivity on pyramidal neurons and granule cells of the dentate gyrus; occasional presynaptic axons are also found here (Romano et al, 1995). Parallel staining with antisera against mGluR1 showed staining of hippocampal interneurons, as described by Martin et al (1992) but no axonal staining. In the hypothalamus, one of the regions showing strong mGluR5 staining is the suprachiasmatic nucleus, whereas the supraoptic and paraventricular nuclei in the same tissue show relatively little staining. Electron microscopy of the SCN shows strong membrane staining, and intense staining of patches some of which are, and others that are not, associated with synaptic specializations (van den Pol,1994). Not all SCN neurons show immunoreactivity. Those that are immunoreactive show labeling predominantly on the inner surface of the plasma membrane, in keeping with the likely position of the C-terminal of this G-protein coupled receptor.

Baude et al (1993) suggested in a recent paper on the hippocampus that mGluR1 staining was found preferentially in perisynaptic regions, that is, at the edges of synapses. If this is true of related metabotropic receptors, one would expect to see the same staining patterns with mGluR5. Our preliminary results with mGluR5 are not consistent with this. This may reflect a focus on a different brain region (hypothalamus) or different experiment approach (immunoperoxidase vs immunogold). To determine if non-synaptic membrane labeling in the SCN might be specific, we examined mitral cells in the olfactory system that do not have asymmetrical synapses on secondary dendrites typical of glutamatergic axonal input. Dendrites of these cells showed strong immunoreactivity at non-synaptic membrane, suggesting that similar labeling in the SCN would not be simply a result of diffusion of the immunoreaction product from a synaptic source (van den Pol,1995).

3. Identification of functional glutamate receptor subtypes in developing hypothalamic neurons.

In addition to identifying glutamate receptor subtypes with antibodies and RNA probes, we have also employed functional examinations, based on Ca2+ digital imaging and patch clamp electrophysiology.

Work with cultured hypothalamic cells and with hypothalamic slices has suggested that glutamate receptors may be functionally expressed in the developing hypothalamus (van den Pol, Wuarin & Dudek,1990; van den Pol, Finkbeiner & Cornell-Bell,1992; van den Pol and Trombley,1993). To address the question of whether glutamate receptors were functionally active in embryonic hypothalamic neurons, Ca^{2+} imaging was used as it is a sensitive indicator of functional glutamate receptor activation in single cells, and because digital Ca2+ imaging allows the recording and analysis of a large number of cells simultaneously. Whole cell patch clamp recording was used as a very sensitive direct indicator of membrane currents elicted by glutamate receptors, independent of the Ca2+ signal. A total of 10,922 hypothalamic neurons were studied with digital Ca2+ imaging with the ratiometric dye fura-2 to examine their responses to glutamate receptor agonists and antagonists during embryonic development and maturation in vitro. These cultures included, but were not restricted to, the SCN. Functional glutamate receptors were found very early in development (embryonic day 15-E15) with both Ca2+ imaging and with patch clamp recording. This is a time when the hypothalamus is beginning to undergo neurogenesis. Ca2+ responses from NMDA receptors developed later than those from non-NMDA ionotropic receptors that responded to kainate and AMPA. The responses of immature E15 cells after 1 day in vitro (1 DIV) were compared with more mature cells (E15/ 6 DIV) to examine the response to repeated 3 min applications of 100 uM kainate (n=108). Immature cells showed similar Ca2+ rises (+232 nM Ca2+) with each kainate stimulation. In contrast, more mature cells showed an initial Ca2+ rise of 307 nM, with the

second rise only to 147 nM above the initial baseline. Immature cells more quickly returned to their pre-kainate baseline than did older cells.

The expression of metabotropic glutamate receptors was studied with the selective agonist t-ACPD and with glutamate stimulation in the absence of extracellular Ca2+ and in the presence of 1 mM EGTA. E16 neurons after 5 DIV or less that responded to 100 uM NMDA showed relatively little indication of metabotropic receptors, whereas cells with a flat astrocyte morphology did not respond to NMDA but showed strong responses indicative of metabotropic receptor activation. A dramatic increase in the percentage of cells that responded to NMDA was found after only a few days in culture. Only a small number of E15 cells studied on the day of culture (4% of 694 cells) showed a response to 100 uM NMDA. 38% of 120 E18 cells cultured for 1 DIV showed an NMDA response. By 4 DIV 95% of 180 E18 cells with a neuronal morphology responded to NMDA; similar results were obtained with 97% of 120 cells that responded after 11 DIV. These results suggest that by the day of birth (after E22) almost all neurons probably already have functional NMDA receptors. To examine the ontogeny of neuronal responses to endogenously secreted glutamate, the decrease in intracellular Ca2+ was studied in response to ionotropic glutamate receptor blockers DL 2 amino 5 phosphonopentanoic acid (AP5) (100 uM) and cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) (10 uM). By 3 DIV, 2 of 128 (1%) E15 cells showed a decrease. By 4 DIV, this proportion increased to 16% of 192 neurons. After 5 DIV most neurons (78% of 192) decreased Ca2+ in response to glutamate receptor antagonists. These data suggest that by 5 DIV, hypothalamic glutamatergic neurons secreted sufficient glutamate from presynaptic axons to elevate Ca2+ levels in the majority of neurons.

Forty-six neurons were studied with whole cell patch clamp recording from E15, E17, and postnatal day 2 (P2) rats within a few hours of plating. In E15 neurons, inward current was seen in response to kainate (100 uM), AMPA (30 uM), NMDA (100 uM), and glutamate (100 uM), with 50% or more of the neurons showing responses to kainate, AMPA, and glutamate; a smaller proportion responded to NMDA. Larger currents were evoked and a higher percentage of neurons (100%) responded to glutamate and its agonists if recordings were made from older P2 hypothalami. Even at the earliest age (E15), GABA (30 uM) evoked large currents from all hypothalamic neurons examined. These data were published this month (July,1995: van den Pol et al,1995).

Taken together these extensive data indicate that functional glutamate receptors are expressed early in hypothalamic embryonic development, at a time prior to synapse formation. At this early stage of development, glutamate induces intracellular Ca2+ increases sufficiently large to potentially influence many factors that play a role in neuronal development, including gene induction, neurite extension, enzyme regulation, and synaptogenesis.

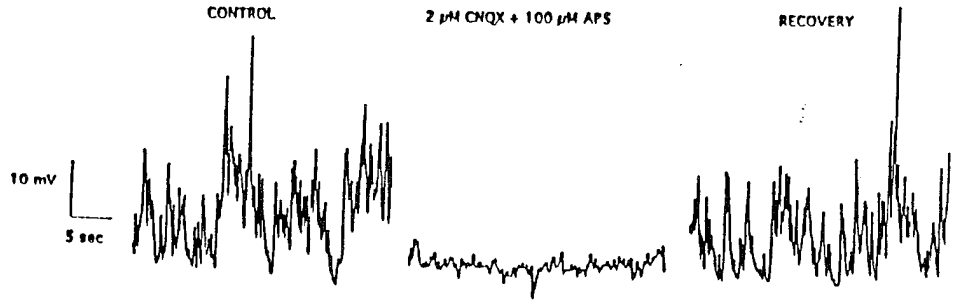
Why is glutamate important during neuronal development? A number of studies have indicated that glutamate can play an important role in developmental plasticity. The speed and direction of growth of cultured neurites is influenced by the application of glutamate (Mattson et al,1988). A significant part of this modulation of neuritic outgrowth may depend on glutamate receptor mediated changes in intracellular Ca2+ (Mattson & Kater,1987). Synaptogenesis and axonal branching are also influenced by glutamate receptors, as studied in retina ganglion cells (Bodnarenko and Chalupa,1993), cerebellum (Rabacchi et al, 1992), somatosensory cortex (Schlaggar et al, 1993), and superior colliculus (Constantine-Paton et al,1990). Migration of neurons during early development is regulated by glutamate, as shown in cerebellar granule cells (Komuro and Rakic,1993). Glutamate, to a large degree acting through Ca2+, also plays a critical role in modulating gene expression (Vaccarino et al,1992; Bading et al,1993). Early responses to glutamate in the SCN may play a role in facilitating the correct development of the glutamatergic retinal projection to postsynaptic neurons in the SCN.

We have studied the Ca2+ response to glutamate agonists in cultures and slices of the SCN. Both neurons and astrocytes showed increased intracellular Ca2+ levels and oscillations of Ca2+ in the continuing presence of glutamate, kainate, and quisqualate. Neurons also responded to aspartate and NMDA in the absence of Mg^{2+}, but astrocytes did not. Astrocyte responses to glutamate indicated Ca2+ waves moving from one astrocyte to another, probably via gap junctions between the astrocytes (van den Pol et al,1992). These results suggest that, similar to the hippocampus (Cornell-Bell et al,1990), astrocytes can be involved in long distance intercellular communication.

4. Glutamate is the primary excitatory transmitter released by hypothalamic neurons; little evidence of SCN cells releasing glutamate was detected.

We have undertaken a series of converging experiments to test the hypothesis that glutamate is an endogenous transmitter of the medial hypothalamus. In contrast to the prevailing belief that hypothalamic neurons use peptides, neuromodulators, or other slow-acting agents as their principal neuroactive substances, our data indicate that the primary excitatory transmitter released by medial hypothalamic neurons is glutamate. This evidence is based on three converging approaches: Immunogold cytochemistry revealed that, in vitro, some hypothalamic neurons and their processes contained high amounts of immunoreactive glutamate. Ca2+ digital video imaging showed that cytoplasmic Ca2+ levels of cultured neurons, elevated because of spontaneous presynaptic release of a hypothalamic transmitter, were reduced by perfusion with the selective glutamate receptor antagonists cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) and 2-amino phosphono-valeric acid (AP5). Electrophysiological analysis of whole cell patch clamp recordings from single and pairs of monosynaptically coupled hypothalamic neurons in culture showed that virtually all spontaneous and evoked excitatory postsynaptic potentials appear to be mediated by synaptic secretion of glutamate, and can be blocked by AP5 and CNQX (Fig.1) (van den Pol and Trombley,1993). Addition of antagonists to other transmitters had little detectable effect.

FIG.1



These experiments utilize neurons from the mediobasal hypothalamus and include the SCN. To test whether SCN neurons show similar properties to the mediobasal hypothalamus as a whole, we cultured SCN neurons, and neurons from the arcuate nucleus as controls. With both digital calcium recording, and with patch clamp electrophysiology, we found that many cells of the arcuate nucleus released glutamate. In striking contrast, when we repeated the same experiments with SCN neurons, we found little evidence of release of excitatory transmitters. We did find many inhibitory postsynaptic potentials that were blockable by 50 uM bicuculline, suggesting the release of the inhibitory amino acid GABA. Also interesting, when we included with our SCN cultures the area immediately surrounding the SCN (+200 um from the border of SCN) we found a striking increase in release of excitatory transmitters. These data suggest that most of the neurons within SCN are inhibitory, and use GABA, but that just outside the SCN are a number of excitatory neurons that use glutamate as a transmitter.

Our initial studies with calcium imaging and patch clamp recording were done within three weeks of culturing the neurons. To test whether a longer period in culture might reveal other endogenous excitatory transmitters, we raised hypothalamic neurons in culture for up to 110 days, more than twice as long as it takes a rat to reach sexual maturity. Our results with fura-2 imaging of these cultures indicated that these older cultures showed more activity, and more Ca2+ spikes and transients. When specific glutamate receptor blockers were added, Ca2+ spikes and transients were completely blocked, and the general Ca2+ baseline was lowered. Blocking activity with tetrodotoxin gave similar results. Together these data indicate that regardless of how long cells are grown in culture, glutamate appears to account for almost all of the excitatory activity (Obrietan and van den Pol, 1995).

5. Excitotoxicity and Ca2+ responses to glutamate.

To study the possible excitotoxicity of glutamate released from hypothalamic neurons, we cultured hypothalamic neurons for over 100 days either in the presence of glutamate receptor blockers CNQX and AP5, or in control cultures lacking the glutamate blockers. Glutamate and glutamine were not added to the culture medium. At regular intervals we compared the responses of control cultures and cultures raised in

the presence of glutamate receptor antagonists. In the control cultures, we found a steady decrease in the number of neurons with time; compared with hypothalamic neurons raised in chronic glutamate receptor block, 50% more neurons died in the control cultures over a period of one month. However, when the glutamate receptor blockers were removed from neurons that had been chronically blocked with glutamate receptor antagonists, 40% of the neurons died within two days, as measured with trypan blue exclusion and with the fluorescent "Live-Dead" dye from Molecular Probes. Cell death could be prevented by the re-introduction of the glutamate receptor blockers within minutes of initial removal. Calcium imaging with fura-2 showed a large and abnormal Ca2+ rise reaching 1000 nM or higher. If the glutamate antagonists were re-introduced within a few minutes, there was little cell death, but if reintroduced several hours later, most cells were unable to reduce their Ca2+ and return to normal baselines of 50-100 nM. A small number of cells showed only a modest Ca2+ response to removal of the glutamate receptor antagonists. These cells were the most likely to survive after glutamate exposure. Cultures containing high percentages of SCN cells were less likely to show a toxic effect to relief from chronic glutamate receptor block due to the low number of glutamatergic cells in such cultures.

6. Glutamate exposure alters expression of its receptors.

A number of studies have shown that transmitters and their receptors may show circadian rhythms of expression in the SCN. A circadian rhythm of glutamate receptor expression may underlie some of the rhythmical responses to glutamate. One of the factors that may alter receptor expression is the diurnal variation in glutamate released by retinal ganglion cells in the SCN. Glutamate receptor activity may influence the synthesis of additional receptors (Bessho et al,1993,1994; Monyer et al,1994). One can add glutamate to cultured cells to determine if glutamate receptors would be down-regulated, but additional glutamate can be toxic to the neurons and cell death becomes a significant problem. Instead, we took the opposite tack and blocked glutamate receptor activity. We had previously shown that cultured hypothalamic neurons release glutamate at synaptic endings on other hypothalamic neurons. By adding AP5 (an NMDA receptor antagonist) and CNQX (a non-NMDA receptor glutamate receptor antagonist) we could block ionotropic glutamate activity. We then studied these neurons in two ways: calcium digital imaging with fura-2 to examine the responses to glutamate and its agonists, and with molecular biology to determine if there was an increase in RNA coding for glutamate receptors using Northern blot analysis.

As indicated above, neurons grown in the presence of AP5 and CNQX showed a heightened response to removal of the glutamate receptor blockers. To test whether this may have been due to an exaggerated response at the receptor, rather than excessive transmitter release, we compared the response of cells to direct application of several glutamate agonists, including kainate, AMPA, NMDA, and t-ACPD (a selective metabotropic glutamate receptor agonist). These experiments were done in the presence of TTX to eliminate release of endogenous transmitters. Cells that were raised in the presence of glutamate receptor blockers showed much greater Ca2+ responses to application of glutamate agonists than did the controls. These exaggerated responses were even seen in the presence of voltage activated Ca2+ channel blockers, suggesting an elevation of glutamate receptors. This idea gained further support from the fact that the lowest levels of response to glutamate agonists were consistently found in the control groups. In hundreds of control and chronically blocked neurons, the 17% (average) with the lowest response were exclusively from the control group, suggesting an up-regulation of glutamate receptors in the cells raised in the presence of glutamate receptor antagonists (Obrietan and van den Pol, 1995).

In addition to examining the physiological responses of cells to glutamate receptor agonists after long term exposure to glutamate receptor antagonists, we (van den Pol and Ghosh, in progress) have studied changes in glutamate receptor gene expression with Northern blot analysis. Hypothalamic and hippocampal neurons were grown in flasks, and treated with combinations of AP5, CNQX, or used as non-treated controls. RNA was isolated, and equal amounts (10 ug) of RNA were run in the same gel. Experiments were run in duplicate or triplicate. Control probes for actin and the 18S ribosomal RNA were used to ensure equal loading in each lane of the gel; these were used after stripping the blot and reprobing with control cDNAs. Combinations of AP5 and CNQX resulted in a dramatic increase in ionotropic glutamate receptor expression, including both NMDA and non-NMDA receptors.

In parallel experiments we have used small punches from 300 um slices of the SCN and used reverse transcriptase to convert RNA to cDNA, and then used PCR with primers unique to different glutamate

receptors. One of the interesting findings of these studies is that the recently cloned metabotropic receptor mGluR7 shows strong expression in the SCN, but not in control regions.

To determine if glutamate receptor block would also increase the response to glutamate in SCN astrocytes, we raised astrocytes in the presence of 1 mM kynurenate to block ionotropic glutamate receptors and in control media not containing glutamate receptor blockers. After two weeks in culture, we compared the dose response curves of SCN astrocytes. SCN astrocytes that were raised in the presence of chronic glutamate receptor block showed a greater sensitivity to glutamate than unblocked control sister cultures (Haak,Heller, and van den Pol, in progress)..

7. Excitatory effects of GABA on developing SCN neurons.

GABA is found in most if not all neurons in the SCN, and is also found in most other brain regions. It is generally thought of as an inhibitory transmitter that gates Cl- channels, allowing Cl- to enter the cell from extracellular space. In mature neurons this results in a hyperpolarization, a decrease in action potentials, and a decrease in cytosolic calcium. In striking contrast, when we examined the responses to GABA of young SCN neurons in vitro, we found that about 75% showed an **elevation** of calcium in response to GABA (Fig.2). We studied the mechanism of this elevation and found that the Ca2+ elevation could be blocked with Cd+ and with nimodipine, a L-type voltage channel blocker, suggesting that GABA was depolarizing young neurons and increasing cytosolic Ca2+ by opening plasma membrane L-channels (Obrietan and van den Pol,1995).

FIG.2

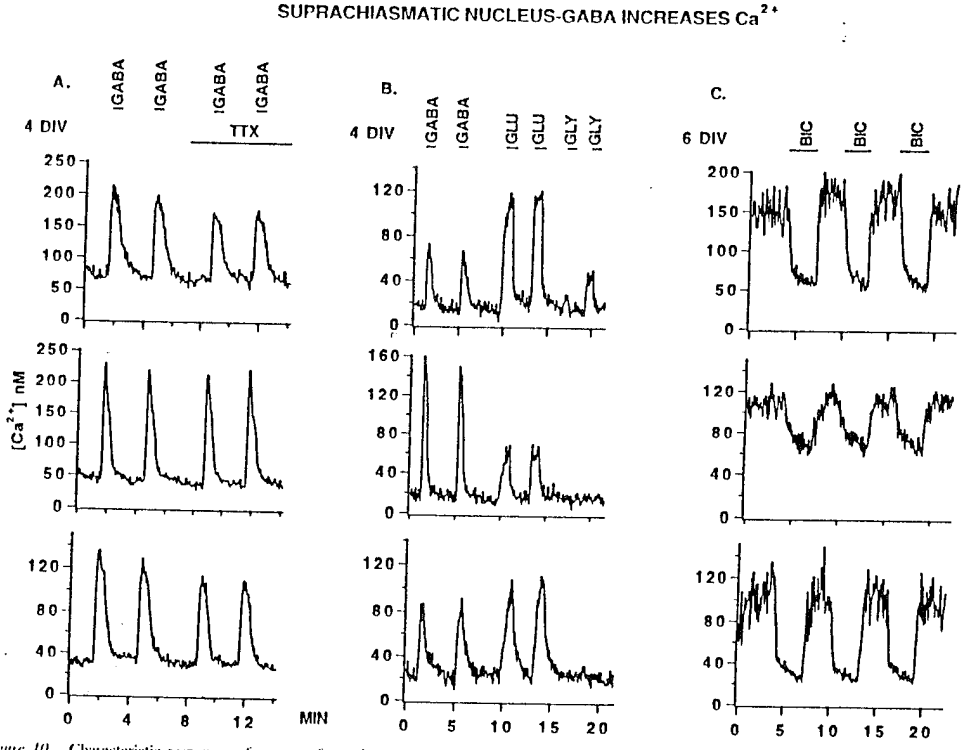


Figure 10. Characteristic responses of neurons from the suprachiasmatic region of the hypothalamus are shown. *A*, Both in the absence and presence of TTX (1 μM), Ca^{2+} levels increased in response to 20 μM GABA, 4 DIV. *B*, Equimolar concentrations of GABA, glutamate, and glycine (20 μM) evoked reproducible increases in Ca^{2+}, 4 DIV. *C*, The addition of 20 μM bicuculline depressed basal Ca^{2+} levels in neurons constantly perfused with AP5 (100 μM) and CNQX (10 μM) after 6 DIV, indicating that GABA was being released by cells in these cultures.

In subsequent studies we have examined the response of voltage-clamped embryonic neurons to glutamate, GABA, and glycine. Every neuron we studied, even in early stages of hypothalamic development, showed substantial currents (500+ pA) in response to GABA (30 uM). Responses to glycine and glutamate were substantially smaller in young neurons, but rapidly increased with age (Chen, Trombley and van den Pol,1995). To determine if these early responses to GABA were excitatory, we used gramicidin perforated whole cell patch clamp recording. Gramicidin allows the voltage or current clamping of a neuron, but as it has relatively little Cl- permeability, we could study the reversal potential

for Cl- without concern that Cl- in the pipette would interfere with the results. We found that the Cl- reversal potential was significantly depolarized in younger neurons (1-7 days) compared with older neurons (17-30 days) (Fig.3). In parallel single channel recording with outside-out patches of young neurons showed GABA-gated Cl- channels with a conductance of 27 pS that reversed at the expected potential for Cl-, at 0 mV in example shown here (Fig.4) with similar pipette and external Cl- concentrations.

FIG.3

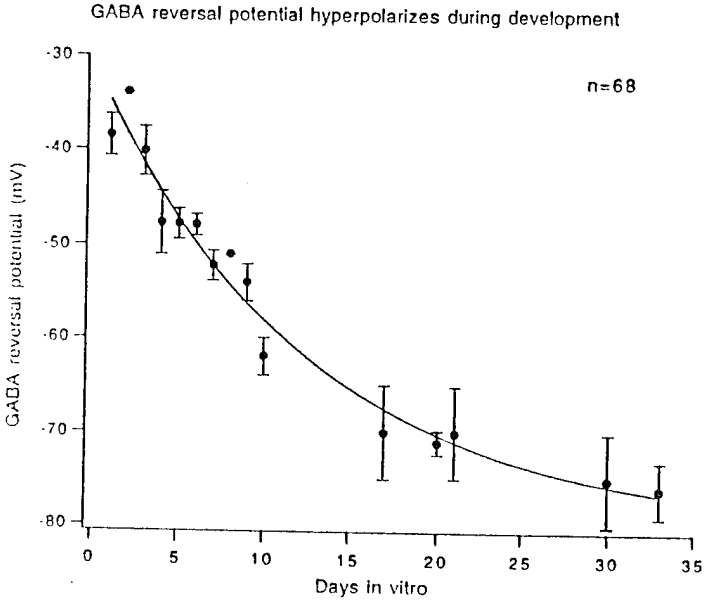
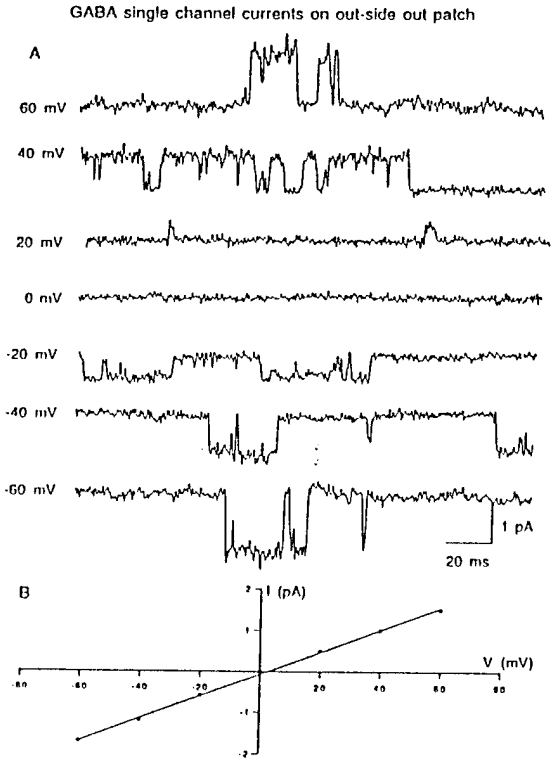
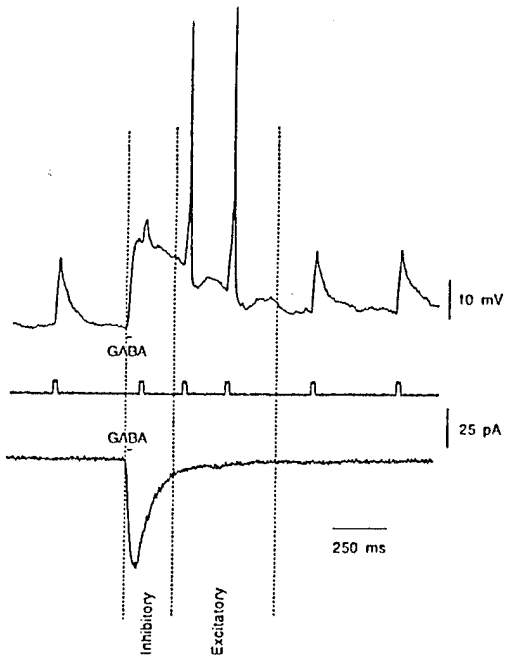


Fig.4



Because the reversal potential for Cl- was depolarized in younger neurons, GABA would act to depolarize these young neurons, but hyperpolarize older ones. A slight depolarization induced by GABA could have one of two effects on neuronal excitability. It could reduce input resistance, shunt current, and still serve to reduce activity, or it could depolarize a cell to the threshold for action potentials, and increase activity. We found that in brief (10 ms) pulse of GABA, the initial effect was one of depolarization, and of current shunting. However, input resistance rapidly increased while the cell was still depolarized. In this later period, current injections (8 pA) elicited action potentials, whereas similar current injections during the period of low input resistance did not generate action potentials (Fig.5). These data suggest that GABA can play both an excitatory or inhibitory role depending on the timing of other inputs. We also found that application of GABA to young cultures often resulted in an increase in action potentials, whereas when GABA was applied to older cultures (14 days+) it reduced activity. Taken together, these data suggest that in young SCN neurons, GABA could exert an excitatory effect.



8. Interactions of glutamate with neuromodulators.

Dopamine modulation of glutamate actions- Ca2+ imaging. To examine the possible modulation of glutamate by neuroactive substances found in the SCN, fura-2 digital Ca2+ imaging was used. Dopamine is synthesized by a number of different hypothalamic neuronal groups as we have described previously (van den Pol et al,1984; van den Pol,1985), and its receptors are strongly expressed in the SCN in developing rodents. When dopamine (10 uM) was applied by itself to hypothalamic neurons in our microscope perfusion chamber, little effect was seen. In contrast, when glutamate (10 uM) was applied, there was a rapid elevation in intracellular Ca2+. Of interest, when dopamine and glutamate (10 uM) were applied simultaneously, the Ca2+ rise generated was significantly greater than found with glutamate by itself. This suggests that dopamine acted to amplify the Ca2+ response to glutamate. When we added dopamine to cultures showing high levels of endogenous activity, we found both with fura-2 calcium imaging, and with whole cell patch clamp recording, that dopamine enhanced activity levels in some neurons, and in others a decrease in activity was found. Dopamine had no effect on activity in the presence of glutamate receptor antagonists AP5 (100 uM) and CNQX (10 uM), underlining the significant role of dopamine in modulating glutamate. We are currently examining the effect of dopamine on postsynaptic GABA responses and on modulation of GABA release (Belousov and van den Pol,1995). These studies are ongoing.

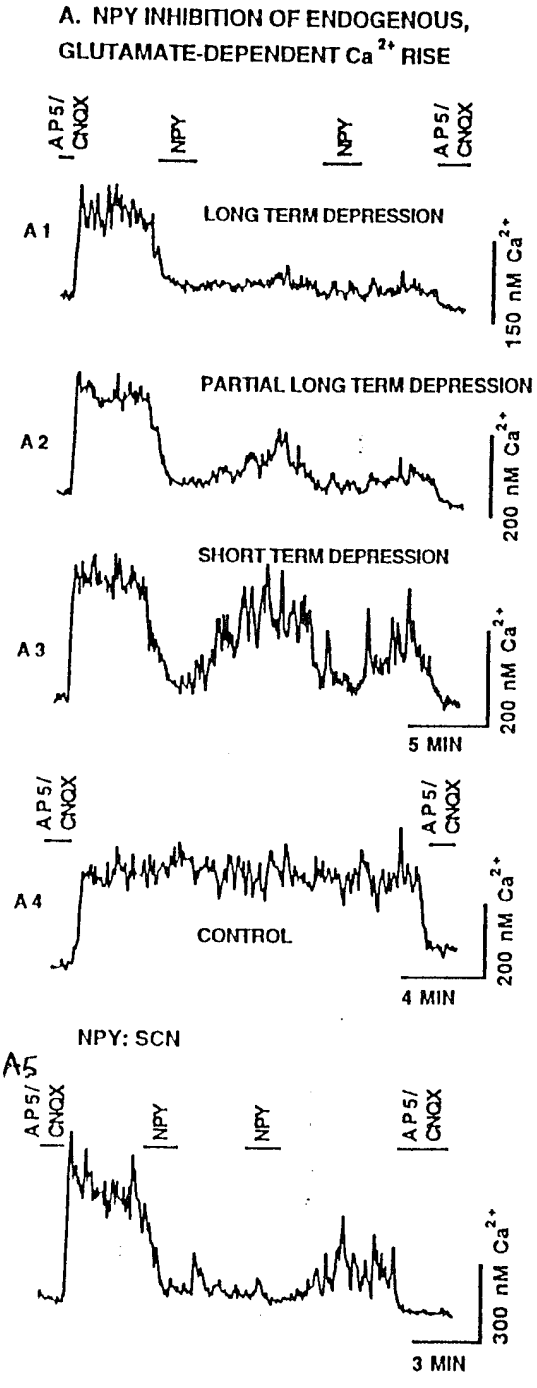
Adenosine modulation of glutamate. Adenosine is released both by neurons and by astrocytes, and my colleague Dr.Heller has postulated that adenosine may be a primary factor in the brain whose activity mediates sleep. We examined the effect on hypothalamic cultures that included the SCN. We found that adenosine, even at concentrations as low as 5 nM, caused a striking reduction in the activity of cultured neurons, studied with both calcium imaging and with whole cell patch clamp recording. No effect of adenosine on neurons was found at any concentrations if glutamate activity was first blocked with AP5 (100 uM) and CNQX (10 uM). Although adenosine showed little direct effect on neurons, it did generate a calcium elevation in astrocytes, suggesting different mechanisms of action may be operating in neurons and glia. To determine if cultured cells were releasing adenosine, we added several selective A1 or A2 antagonists. A1 antagonists tended to increase activity, and to increase intracellular Ca2+ levels, suggesting that adenosine was being released by the cultured cells, and that it acted to reduce levels of activity of hypothalamic neurons (Obrietan, Belousov, Heller, van den Pol, In press).

9. NPY modulation of glutamate actions- Fura-2 Ca2+ imaging and patch clamp recording- Pilot data

Our preliminary work with fura-2 digital imaging of SCN neurons in vitro stimulated with NPY supports the basic hypothesis of this application. That is that peptides, NPY in this case, exert a significant effect in modulating the actions of the amino acid transmitters, and in the absence of amino acid neurotransmission, little effect of the peptide on cell activity is found. Neurons from hypothalamic cultures that included the SCN and cultures more restricted to SCN and the periSCN area showed robust glutamate-mediated excitatory activity (blockable by AP5 and CNQX). These cells were treated with a brief application of 200 nM NPY. Of 600+ neurons tested so far, 90%+ showed a response to NPY, suggesting that even though some investigators have so far not found strong NPY receptor expression in the hypothalamus, the activity of a large number of neurons is influenced by NPY. About 40% of the neurons tested showed a reduction in spontaneous Ca2+ spikes, and a significant decrease in intracellular Ca2+ in the presence of NPY, that recovered to normal levels upon NPY washout (FIG.6A3). A second large group of neurons (38%) responded in a manner seen only rarely in the course of analyzing the glutamate-related response of over 10,000 hypothalamic neurons, described in several of our previous papers. The initial administration of NPY caused a significant decrease in intracellular Ca2+ that stayed low even long after NPY washout (Fig.6A1). It appeared that NPY reduced the cell's intracellular Ca2+ to a new lowered set point. Although these novel results are preliminary, they suggest that a single relatively brief application of NPY can have a long-lasting effect, far exceeding the duration of NPY presence. Some of these cells took up to an hour to show complete recovery to normal Ca2+ levels. Of considerable interest, we also found a group of neurons (5%) that showed enhanced activity in the presence of NPY in the same medial hypothalamic cultures. We postulate that the enhanced activity may be due to a NPY

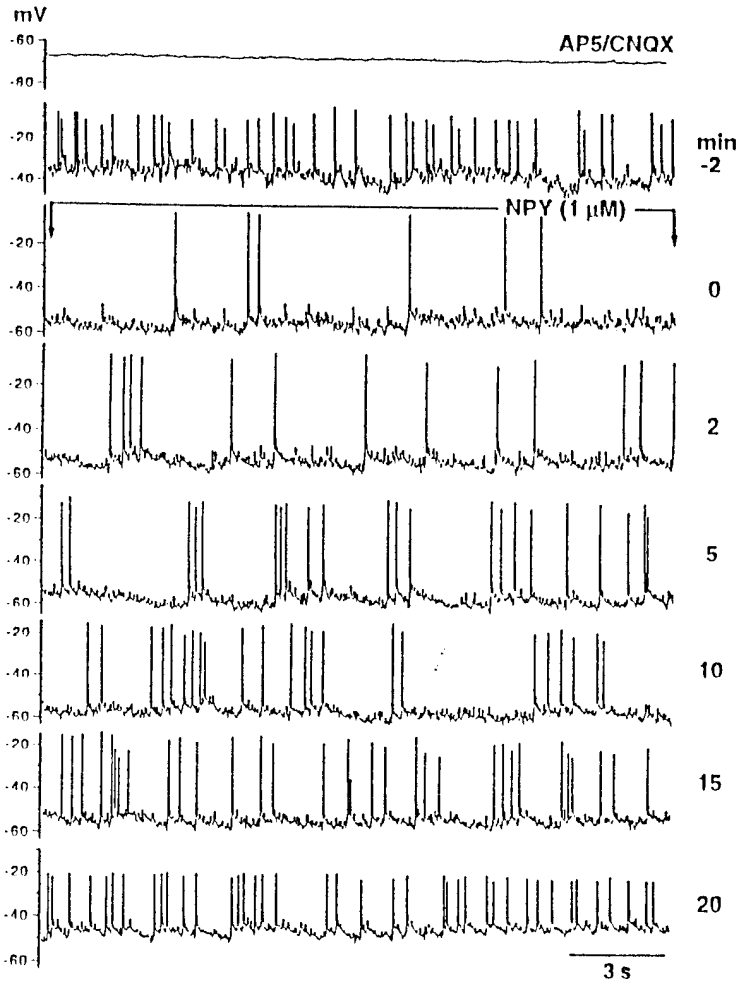
mediated decrease in GABA release from hypothalamic neurons. In control experiments (not shown here) NPY had no effect on Ca2+ levels if glutamate neurotransmission was blocked with AP5 (100 uM) and CNQX (10 uM), strongly supporting our hypothesis that a major role of NPY is to modulate glutamate activity.

FIG.6. Glutamate is spontaneously released from presynaptic neurons in this hypothalalmic culture. In a single culture NPY caused in Neuron A3 a decrease that recovered upon NPY washout, Neuron A1 a long term depression in Ca2+, Neuron A2 a partially extended depression of intracellular Ca2+.
Neuron A4 is a control showing no change in Ca2+ activity in the absence of NPY.
A5 is a neuron from the SCN that show the long term depression of intracellular Ca2+ upon application of NPY.

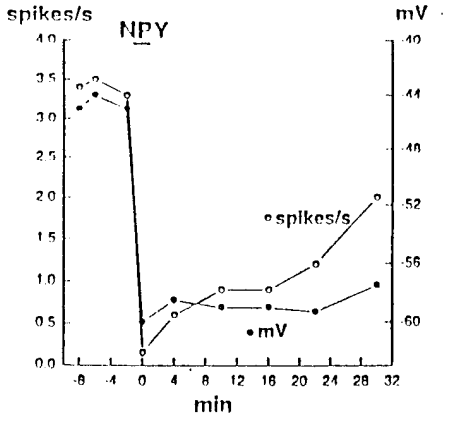


In parallel, when NPY was perfused on a neuron in culture, the spike frequency and membrane potential were strongly reduced. This is shown in an example from a single cell in Fig.7 to the right.

FIG.7. Patch clamp recording of neuron before, during, and after NPY application. Glutamate receptor blockers AP5 (100 uM) and CNQX (10 uM) blocked activity. NPY (1 uM) caused a strong reduction in action potentials, and a membrane hyperpolarization.

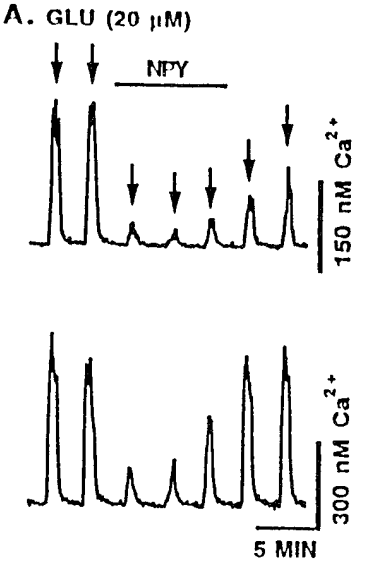


Membrane potential and spike activity changes in the presence of NPY are shown in this line graph of a second neuron below (Fig.7B).



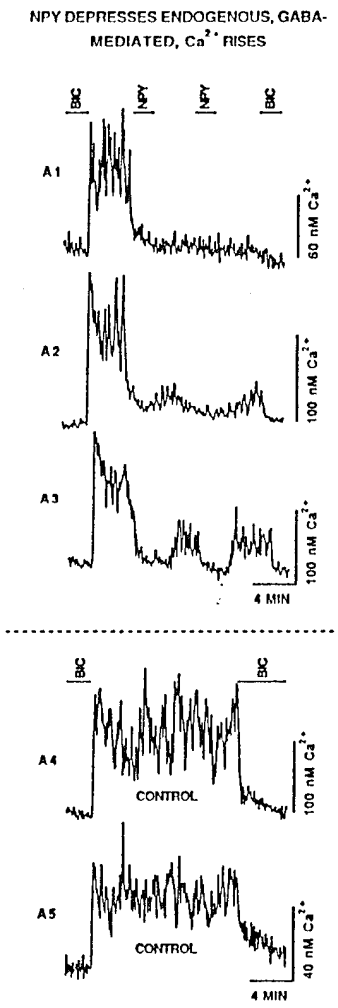
We also tested the possibility that NPY would exert a postsynaptic effect on glutamate mediated Ca2+ rises in hypothalamic cells, studied with fura-2 digital imaging. The experiments were done in the presence of 2 uM tetrodotoxin to block endogenous transmitter release, and thereby eliminate a presynaptic effect of NPY. A striking reduction in the Ca2+ response to 20 uM glutamate was caused by 100 nM NPY. In Fig.8 below, NPY caused a typical reduction in the response of the neuron to glutamate. Times of application of glutamate or NPY are shown by the horizontal bar above Ca2+ record. **These data suggest strongly that NPY actions in hypothalamic neurons, including SCN neurons, are quite different from those in hippocampal or raphe neurons,** where several well-done experiments indicated that NPY had no effect on postsynaptic glutamate-mediated Ca2+ responses (Bleakman et al,1992).

FIG.8. Glutamate (20 uM) at arrows causes increase in intracellular Ca2+ in 2 cultured neurons. NPY administration (at time marked by horizontal NPY bar) significantly reduced the neuron's response to glutamate by a postsynaptic mechanism. Almost all neurons (98%) showed full recovery as soon as NPY was washed out, as represented by lower cell. Only rarely (<2%) was there a lingering effect after NPY washout. This is in contrast to NPY application to cells showing endogeous glutamate activity (i.e. Fig.6 above) where a large number (38%) showed a long term depression.



10. NPY modulates GABA activity in SCN.

In Section 7 of this progress report, the ability of GABA to increase intracellular Ca2+ in young cells was examined. We have tested the hypothesis that Ca2+ elevating activity of GABA would be modulated by NPY. In initial experiments, we found that NPY, which had little effect by itself, could dramatically reduce calcium levels elevated by GABA in young SCN cells (Obrietan and van den Pol, in progress). The $GABA_A$ receptor antagonists bicuculline (30 uM) eliminated Ca2+ transients in these cells, and blocked any NPY effect. Because these experiments were done in the presence of glutamate receptor blockers AP5 (100 uM) and CNQX (10 uM), it is unlikely that the effects in this experiment were due to glutamate modulation by NPY. These initial observations (n=100+) suggest that GABA activity in the SCN can be modulated by NPY, and that this modulation can occur even in early stages of development. These experiments also raise interesting questions that we plan to pursue in the present application, such as whether NPY modulation of GABA is pre- or postsynaptic, and what the mechanism is for NPY reducing GABA's effect.

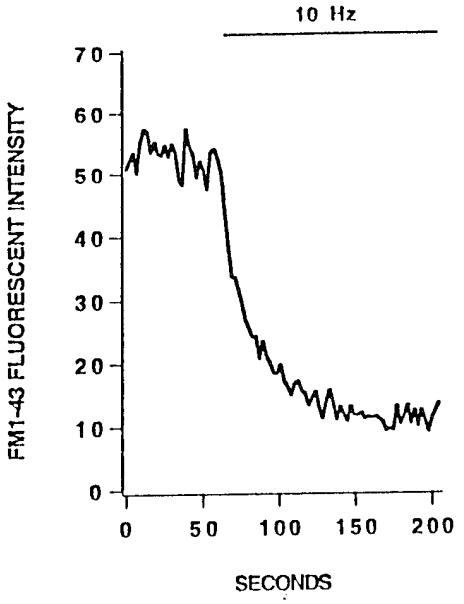


11. VGF expression in hypothalamus and brain.

In 1989 we described a new protein expressed in the brain, based on an antibody made from bacterially produced protein transformed with the gene VGF (van den Pol et al,1989). With immunocytochemistry, the expression of this protein was strongest in the SCN. We have extended our initial observations to other regions of the brain where immunoreactivity is less intense than in the hypothalamus. Immunoreactivity was found in the hippocampus, striatum, cortex, and septum. The intensity of immunostaining in non-hypothalamic regions was less than seen in the same histological sections in the hypothalamus. Of interest, when we compared the relative levels of gene expression in Northern blots, we found that contrary to our expectation, the hypothalamic lanes showed less RNA than hippocampus, cortex, or other regions of the brain. Control hybridization with actin and 18s probes showed equal RNA loading in all lanes. These data suggest that either the protein is metabolized rapidly in other regions of the brain, or that the efficiency of translation from RNA into protein is greater in the hypothalamus, and particularly the SCN, than in other brain regions (van den Pol et al,1994).

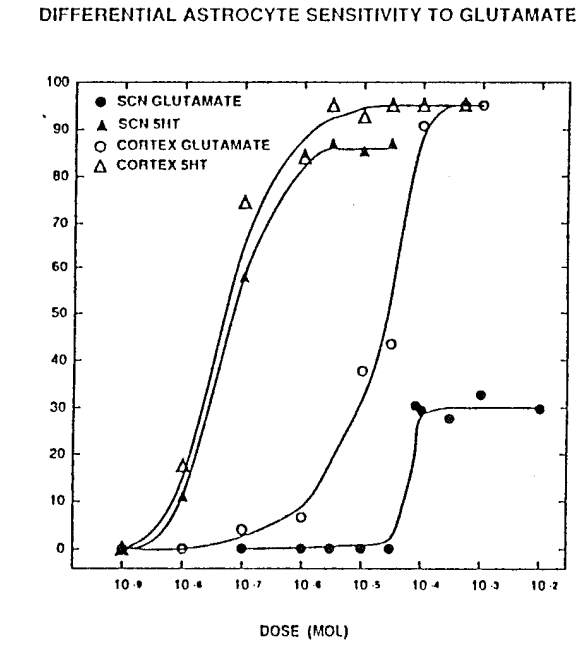
12. Vesicle exocytosis detected with the dye FM1-43.

During release of glutamate and GABA, small clear synaptic vesicles fuse with the axonal membrane, release transmitter, and are then recaptured from the membrane. The dye FM1-43 is a relatively new fluorescent dye that can be used to study exocytosis and membrane recycling (Ryan et al,1993; Ryan and Smith,1995). In our experimental proposal we plan to use this dye to study NPY modulation of vesicle exocytosis in culture, particularly to examine presynaptica effects of NPY. Here we demonstrate that after loading the dye, we can then study exocytosis in a single axon terminal by stimulating the axon to fire action potentials at 10 Hz for 200 sec. The fluorescence signal from FM1-43 is rapidly decreased to baseline levels, indicating vesicle exocytosis and transmitter (and dye) release from this single bouton of a developing neuron. In controls, if action potentials are blocked with 200 nM TTX, no change in fluorescence is found with stimulation.We postulate that NPY will reduce the release of transmiter (see Experimental Proposal. FIG.9



13. Differential responses of SCN and cortical astrocytes to neurotransmitters. FIG.10

We have shown that SCN astrocytes respond to two of the major transmitters innervating the SCN, glutamate and serotonin (van den Pol et al,1992). Both have been implicated in regulation of phase shifts of the circadian rhythm. Although we commonly consider neurons to be the primary cell involved in regulation of circadian rhythms, astrocytes may also play a role. Recent reports demonstrating astrocytes communication to neurons have underlined the possible functional importance of astrocytes as playing a larger role than the traditional role of structural support and nutrition for neurons. In the course of studying astrocyte calcium responses to serotonin and glutamate, we found some very interesting differences between astrocytes from the SCN and from other brain areas. For instance, dose response curves demonstrate that SCN astrocytes are much less responsive to glutamate than cortical astrocytes, but that their response to serotonin is very similar to cortical astrocytes (Fig.10). Whereas glutamate is considered to be an excitatory transmitter, and one that raises cytosolic calcium levels, in SCN glia we found that glutamate could actually play a depressing role, reducing cytosolic calcium levels that had been raised by stimulation with serotonin (Haak et al,in press). Furthermore, cellular oscillations initiated by serotonin could be dramatically altered by the addition of glutamate, even in neurons that showed no oscillations in direct response to glutamate alone. This work is ongoing and is part of the dissertation work of Laure Haak done with Dr.van den Pol's fura-2 rig at Stanford under the co-direction of Drs. Heller and van den Pol.



5. Key personnel whose work was supported by AFOSR (1995).

Anthony N. van den Pol,PhD
Andrei Belousov,PhD\ Postdoctoral fellow
Karl Obrietan, Graduate student
Laure Haak, Graduate student
Vinh Cao, Research Associate

6. Publications.

Relevant papers completed in the last year (1994/95). A total of 25 papers were completed during this period. Some papers during this period that are not listed below are found on the PI's CV, as are other papers on SCN supported by the AFOSR in previous years.

van den Pol, A.N., I. Hermans-Borgmeyer, M. Hofer, P. Ghosh, and S. Heinemann (1994) Ionotropic glutamate-receptor gene expression in hypothalamus localization of AMPA, kainate, and NMDA receptor RNA with in situ hybridization. Journal of Comparative Neurology 343: 428-444.

van den Pol, A.N., C. Decavel, K. Bina, and P. Ghosh (1994) VGF expression throughout the brain. Journal of Comparative Neurology 347: 455-469.

van den Pol, A.N., L. Kogelman, P. Ghosh, P. Liljelund, and C. Blackstone (1994) Developmental regulation of the hypothalamic glutamate receptor mGluR1. Journal of Neuroscience 14: 3816-3834.

van den Pol,A.N. (1994) Metabotropic glutamate receptor mGluR1 distribution and ultrastructural localization in hypothalamus. Journal of Comparative Neurology: 349: 615-632.

Romano,C., M.A.Sesma, C.MacDonald, K.O'Malley, A.N. van den Pol, and J.Olney (1995) Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. Journal of Comparative Neurology: 355: 455-469.

van den Pol,A.N. ,K.Obrietan, V.Cao, and Pl.Trombley (1995) Embryonic hypothalamic expression of functional glutamate receptors, Neuroscience: 67: 419-439.

Obrietan,K. and A.N. van den Pol (1995) Calcium hyperexcitability in neurons cultured with glutamate receptor blockade. Journal of Neurophysiology 73: 1524-1536.

van den Pol,A.N., J.P.Wuarin, and F.E.Dudek (1995) Glutamate neurotransmission in the neuroendocrine hypothalamus. In: Excitatory Amino Acids: Their Role in Neuroendocrine Function, Edited by V.B.Mahesh and D.W.Brann, CRC Press, Boca Raton, FL. In press.

van den Pol,A.N. (1995) Presynaptic metabotropic glutamate receptors in adult and developing neurons: autoexcitation in the olfactory bulb. Journal of Comparative Neurology. 359: 253-271.

Obrietan, K. and A.N.van den Pol (1995) Developmental reversal of the role of GABA in elevating and depressing Ca2+. Journal of Neuroscience. 15: 5065-5078.

van den Pol,A.N., C.Romano, and P.Ghosh (1995) Metabotropic glutamate receptor mGluR5: subcellular distribution and developmental expression in hypothalamus. Journal of Comparative Neurology. In press.

Chen,G., P.Q.Trombley, and A.N.van den Pol (1995) GABA receptors precede glutamate receptors in hypothalamic development differential regulation by astrocytes. Journal of Neurophysiology. In press.

Obrietan,K., A.Belousov, H.C.Heller, and A.N. van den Pol (1995) Adenosine pre- and postsynaptic modulation of glutamate dependent calcium activity in hypothalamic neurons. Journal of Neurophysiology. In press.

Romano,C., A.N.van den Pol, and K.O'Malley (submitted) Developmental regulation of metabotropic receptor mGluR5: mRNA splice variants, protein, and immunocytochemistry.

van den Pol,A.N.,A. Belousov, K.Obrietan (submitted) Glutamate hyperexcitability and seizure-like activity throughout brain and spinal cord upon relief from glutamate receptor blockade in vitro.

van den Pol,A.N. V.Cao, and A.Belousov (submitted) Dopamine modulation of glutamate-regulated calcium and electrical activity in hypothalamic neurons.

van den Pol,A.N. K.Obrietan, G.Chen (submitted) Neuronal trauma and GABA actions.

van den Pol,A.N., K.Obrietan, A.Belousov (submitted) Clock memory: NPY-mediated long term depression of intracellular calcium in the SCN.

Obrietan,K. and A.N.van den Pol (Submitted) NPY depresses GABA-mediated calcium transients in suprachiasmatic nucleus neurons.

Chen,G., P.Q.Trombley, A.N.van den Pol (Submitted) Excitatory electrophysiological actions of GABA in the developing hypothalamus.

Silver,R., G.Block, M.Lehman, T.Ronenberg, A.N.van den Pol, and M.Zatz Cellular substrates for circadian oscillators. In Biological Rhythms. From Physiology to Genetics Edited by J.Dunlap and J.Loros, Oxford Univ. Press, New York.

van den Pol,A.N., J.Strecker, and F.E.Dudek Excitatory and inhibitory amino acids and synaptic interactions in the suprachiasmatic nucleus. Prog.Brain Res submission.

Abstracts.

Bina, K., A.H. Cornell-Bell, and A.N. van den Pol (1993) Metabotropic glutamate receptors regulate calcium in cultured neurons and glia from the suprachiasmatic nucleus. Soc.Neurosci.Abstr. 19: 1815.

van den Pol, A.N., L. Kogelman, P. Ghosh, and C. Blackstone (1993) Metabotropic glutamate receptor gene expression in the developing hypothalamus. Soc.Neurosci.Abstr. 19: 474.

van den Pol, A.N., K. Obrietan, V. Cao, and P.Q. Trombley (1994) Glutamate receptors in embryonic hypothalamus. Soc.Neurosci. Abstr.20: 492.

Haak,L., H.C.Heller, and A.N.van den Pol (1995) Synergistic modulation of calcium by glutamate and serotonin in suprachiasmatic nucleus glia. Soc.Neurosci. Abstr. In press.

Chen,G., P.Q.Trombley, and A.N.van den Pol (1995) Amino acid receptor expression in developing hypothalamus; differential regulation by astrocytes. Soc.Neurosci.Abstr.

van den Pol,A.N. (1995) Excitatory and inhibitory amino acid transmitters in the adult and developing suprachiasmatic nucleus. International Conference on the suprachiasmatic nucleus. Netherlands Brain Research Inst. In press.

Belousov,A. and A.N. van den Pol (1995) Dopamine modulation of GABA neurotransmission in hypothalamic arcuate nucleus neurons. Soc.Neurosci. Abstr. In press.

van den Pol,A.N., K.Obrietan and A.Belousov (1995) NPY modulation of hypothalamic glutamate transmission. Soc.Neurosci.Abstr. In press.

7. Interactions

We have been invited to present the results of experiments supported by the AFOSR at a number of meetings during the last year.

1. International Summer School- Hypothalamic regulation of circadian rhythms- Amsterdam Holland Aug.1995
"GABA and glutamateactions in the suprachiasmatic nucleus that regulate circadian rhythms"

2. Physiological Society Congress of Biological Rhythms- Cellular and molecular substrates.
Dartmouth College- Hanover, N.H. July 1995
"Molecular and physiological analysis of amino acid transmitters contributions to the suprachiasmatic nucleus"

3. Society for Neuroscience Annual Meeting Miami, Fla Nov.1994
"Early action of glutamate"

4. Yale University Medical School Oct.1995
"The circadian clock of the brain, the suprachiasmatic nucleus"

5. Stanford University May,1995
"GABA: excitatory actions in the suprachiasmatic nucleus"

6. Cambridge University, England Sept. 1995
"GABA and glutamate in the hypothalamus and after trauma"

Our closest contact lab in the DOD is the lab of Dr.Michael Rea in San Antonio TX , Brooks Air Force Base, who is working on parallel questions of glutamate activation of immediate early genes in the SCN.

8. No patents were applied for.